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DIVISION OF PATHOLOGY AND PHYSIOLOGY. BULLETIN No. 9

REPORT OF WORK
OF THE
EXPERIMENT STATION
OF THE
HAWAIIAN SUGAR PLANTERS' ASSOCIATION

The Bacterial Flora of Hawaiian Sugars.

BY L. LEWTON-BRAIN AND NOËL DEERR

HONOLULU, HAWAII
APRIL, 1909

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LETTER OF TRANSMITTAL.

To the Experiment Station Committee of the

Hawaiian Sugar Planters' Association

Honolulu, T. H.

Dear Sirs:

I herewith submit for publication an article on the 'Bacterial Flora of Hawaiian Sugars' which has been prepared by Mr. Noël Deerr, Assistant Director of the Division of Agriculture and Chemistry, and myself. As the major portion of the work has fallen on this Division, I would suggest that the article be published as Bulletin No. 9 of the Division of Pathology and Physiology. Broadly speaking, Mr. Deerr is responsible for the chemical work, analyses, etc., while the bacteriological work, inoculations, etc., have been mine.

Yours very truly,

L. LEWTON-BRAIN,

Director, Division of Pathology and Physiology.

Honolulu, T. H., April 25, 1909.

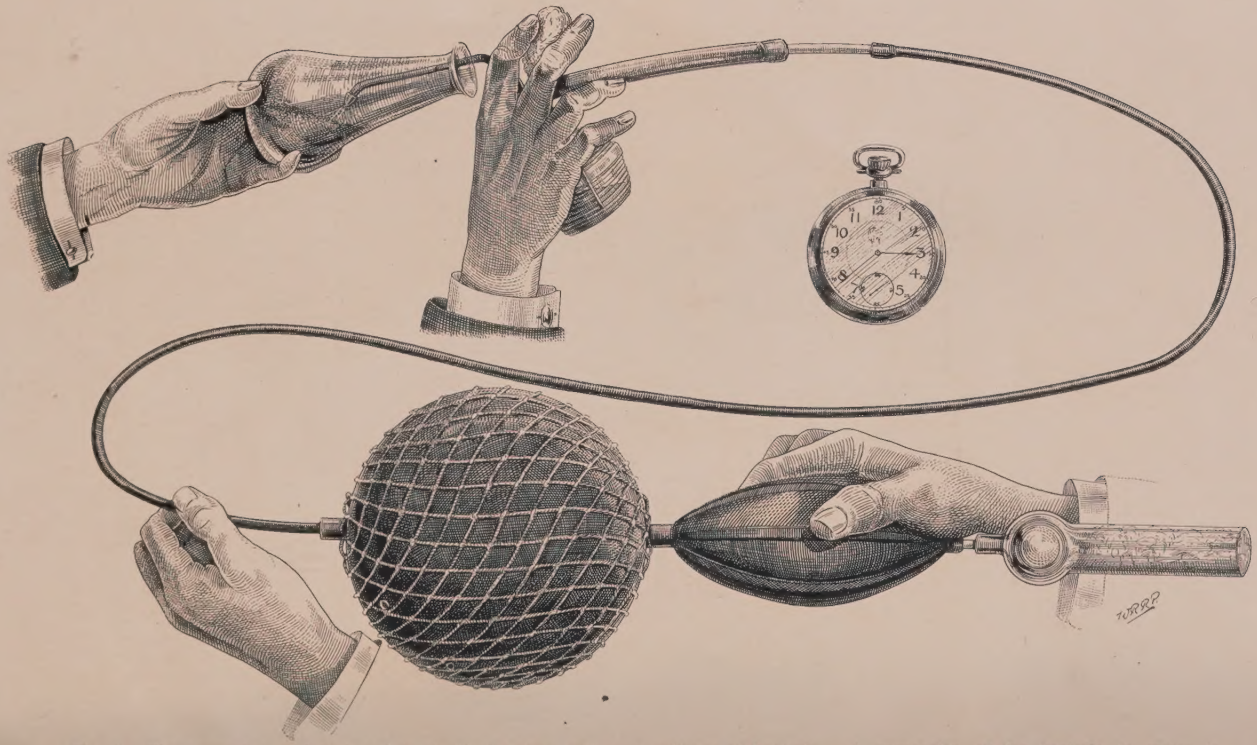

Fig. 1. The apparatus used to inoculate sterilized sugars with pure cultures of bacteria. A suspension of the bacteria in water is pumped from an atomizer into the flask which is turned so that an even distribution is obtained. The air is sterilized by passing through cotton wool, and an even distribution is maintained by keeping up a constant pressure in the second bulb. The apparatus is described on p 8.

THE BACTERIAL FLORA OF HAWAIIAN SUGARS.

By L. Lewton-Brain and Noël Deerr.

The fall in polarization of sugars in storage, and especially in transit on the voyage from Honolulu to New York round Cape Horn, has formed a subject of discussion for the last ten or fifteen years, and is, on its merits, one of great importance and deserving of detailed study, since very considerable sums of money may be annually lost to the producers on this account.

In Bulletin 24 of the Division of Agriculture and Chemistry, one of us in conjunction with Dr. R. S. Norris gave the results of an inquiry into the causes of the deterioration of sugars on storage. Briefly, the results then obtained led to the conclusion that deterioration was primarily to be ascribed to the action of bacteria acting in the presence of a minimum of one per cent. of water. During the course of the above mentioned inquiry, it became evident that certain types of bacteria were of frequent occurrence, and the necessity of determining the source of infection of the raw sugars then became an obvious problem. Before this problem could be attacked, it was necessary to isolate these types of bacteria in pure culture and to obtain their characteristics for the purpose of future investigation. This has now been done, and the present publication is chiefly concerned with an account of the characteristics of the bacteria occurring in Hawaiian sugars. Incidentally, advantage of the opportunity was taken to study the action of each specific organism on commercial sugars with reference to the effect of moisture and to the deterioration caused by each type under equal conditions.

With the data now obtained, it is proposed, as soon as opportunity occurs, to carry on an investigation in typical factories with the object both of determining the several points of infection and of studying the effect of the various routine processes upon the distribution of bacteria in a sugar factory.

Previous work on the deterioration of raw sugars has already

correlated this source of loss with micro-organisms. Shorey* considered that moulds, including the ubiquitous *Penicillium glaucum,* were the chief factors at work. Greig Smith† ascribed the loss to one specific organism which he has described under the name of *Bacillus levaniformans,* which he isolated from a number of sugars of such various origins that he felt justified in considering its distribution as universal. This organism was in particular found by him as a constant inhabitant of juices in Australian sugar factories where it has been observed as the agent through which juices became gummy and viscous.

Grieg Smith does not consider that moulds are to be considered as a cause of the deterioration of sugars, and our work confirms his view in that moulds were but rarely found in the sugars that we have had occasion to examine.

Method of Isolation and Study.

The organisms described in the following pages were isolated from the collection of Hawaiian sugars, the results of the study of which formed the contents of Bulletin 24 of the Division of Agriculture and Chemistry.

Preliminary tests were made to determine the relative abundance of the different bacteria in various raw sugars. A few grains of a sugar that was known to contain a preponderating number of the bacterium required was used to inoculate a dilution series of agar plates. When the last members of a series were approximately pure cultures of the particular bacterium, a further dilution was made from these and carried until only one colony developed in the last plate. From this colony a fresh series was made and if all the colonies developing proved to be identical and of the form required, it was assumed that the bacterium had been obtained in pure culture.

The principal culture medium used for plate and stab cultures was the 10 per cent. sugar, with nutrient salts and peptone, containing 1.5 per cent. agar. Besides this, plate cultures were made on neutral nutrient agar and glucose nutrient agar. The same media were also used for liquid cultures without the agar; litmus sugar solution was used for testing acid production. Potato, carrot and 'turnip' formed the solid media on which the

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* Jour. Soc. Chem. Ind., XVII, 555.

† Proc. Lin. Soc., N. S. W., XXVI, 674, & Int. Sug. Jour., IV, 430.

bacteria were grown; the 'turnip' was a Chinese vegetable and in flavor and texture was more nearly a giant radish than a true turnip.

From the various media transfers were always made back to the sugar agar plates for the purpose of establishing the identity of the bacterium under observation.

It is much to be regretted that gelatin media were not available for these cultures. Attempts had previously been made to produce a gelatin medium in the laboratory of the Pathological Division without success, but for the purpose of this work numerous trials were again made, using different brands of gelatin and also mixtures of gelatin and agar. As high as 30 per cent. gelatin, and 20 per cent. plus 0.75 per cent. agar were used without producing anything but a very viscous liquid at ordinary room temperatures (25° C.). The temperature alone cannot be held responsible, as one of us had previously met with no difficulty in working with 20 per cent. gelatin media in the West Indies,* while a medium containing 12 per cent. gelatin and 0.75 per cent. agar is considered safe for an incubator at 37° C. Every suggested explanation of the failures was disproved by further trials.

Fig. 2. Cells and spores of a "weed" bacillus that continually developed in our intermittently sterilized media. It escaped the sterilizations by completing its growth and producing spores in all its cells within twenty-four hours, so that when the next sterilization took place it had already assumed its resistant form.

Considerable trouble was caused in our earlier work by the spasmodic appearance in various media of what appeared to be a heat-resistant bacterium. It proved to be a form that produced its small colonies and proceeded to spore formation within twenty-four hours, so that it escaped the intermittent sterilization; it most probably was introduced with the water. By thorough dry sterilization of all our glassware and by using only Berkefeld filtered water for all purposes connected with the work, further annoyance was avoided. Such rapidly sporing bacteria are fortunately not of common occurrence and the appearance of one is thought worthy of record. A drawing of this form, as it appeared on some nutrient agar in twenty four hours, is given in Fig. 2.

* Lewton-Brain, West Indian Bulletin, Vol. V, p. 184.

After each organism had been isolated in an ascertained state of purity, its action on sugar in liquid culture and on commercial raw sugar was studied.

For the effect of the bacteria in liquid cultures, we employed a 10 per cent. solution of cane sugar containing in addition 1 per cent. potassium chloride, 0.5 per cent. sodium phosphate, and 0.2 per cent. of peptone. Erlenmeyer flasks containing 100 cc. of this medium were, after sterilization, inoculated with a platinum needle from a pure culture of the organism under examination. They were then kept in an incubator at a temperature of 33° C.; these solutions were analyzed at the end of five days.

The contents of a five day old flask were used to inoculate sugars, the experiment being as follows: About 100 grams of a typical raw sugar were weighed into each of a number of Erlenmeyer flasks which had been plugged with cotton wool and sterilized at a dry heat. The flasks and their contents were then placed in a sterilizer at 100° C., and kept at this temperature for thirty minutes. Numerous efforts were made to obtain a more stringent sterilization, but were all unsuccessful. The trouble was caused by steam condensing and running down the sides of the flasks, whereby the sugar was caked into a solid block. The same trouble occurred when the sugars were subjected to a preliminary drying in a hot air oven and when they were placed in an oven directly on removal from the sterilizer.

We decided, therefore, to adopt one sterilization, taking several flasks and selecting for the purposes of experiment those that showed least caking. It was thought that the control flasks would be a sufficient check on the previous sterility of the inoculated sugars; and in any case, the number of bacteria left would be so small compared with the number introduced in an inoculation that the effect of the bacteria remaining would be practically nugatory. The results showed that this method was thoroughly sound, as in no case was any appreciable deterioration noticed in the control experiments detailed below. After having obtained a number of flasks of sugar containing sterile or nearly sterile sugar, it was necessary to infect them with the pure cultures of the bacteria under examination. The container of an atomizing apparatus, Fig. 1, was filled from one of the flasks containing the fluid culture of one of the micro-organisms. On pumping air into the container by pressure on the bulb, the contents of this were expelled in a fine spray; a constant pressure and hence a constant discharge was obtained through the agency of the second bulb, which was kept at its full tension during the whole time the inoculation is taking place. A preliminary experiment with sterile water

gave the amount of water discharged in one second, and from this datum it was easy to calculate how long to allow the atomizer to work so as to discharge such a quantity of water as would bring the percentage of water in the sugars to a prearranged figure. The container and discharge tubes were sterilized before use and the addition of a tube packed with cotton wool freed the air from any adventitious organisms. A new atomizer was used for each bacterium, so that there might be no chance of contamination with the other sugar bacteria. Using the apparatus in the manner shown in Fig. 1, the sugar contained in the flasks could be inoculated with cultures of the organism under examination and a uniform distribution over the sugar therein contained obtained.

The controls referred to below were treated in exactly the same way with the substitution of sterile water for the bacterial culture, our object being to obtain exactly similar conditions with the exception of the intentional presence of a pure culture of the organism under examination. The controls were, of course, sprayed before the inoculated ones. This method gave a very thorough and uniform inoculation, so that the results of the tests were strictly comparable. To carry it out, at least two operators are necessary, one holds the flasks and guides the spray onto the sugars, while the second devotes his attention to maintaining a steady pressure in the bulbs; the presence of a third person to time the inoculation was found to help considerably. In all the later experiments, both inoculated and control flasks were covered with a piece of thin rubber tissue so as to prevent the drying out of sugars, which was found to result at the temperature of over 30° C. in the incubator.

Culture Characteristics.

By far the best growth, with all the forms except the *Diplobacillus* B, took place on the sugar agar; as the glucose nutrient agar contained an equal amount of available sugar, it would seem that the beef extract had an actual inhibitory effect on these bacteria. With the liquid cultures, also, the growth in the simple sugar-peptone solution was noticeably more vigorous, in the four forms mentioned, than in either the nutrient bouillon or the glucose bouillon.

These facts tend to show that these bacteria are specifically sugar organisms and are not generally distributed forms which happen to have been met with in sugars and sugar factories. This view is strengthened by the proved ability of the five bacteria to grow actively in the thin film of saturated sugar solution as met with in the moistened sugars, where the osmotic conditions must

be such as to preclude any activity on the part of organisms not specially adapted for the environment. The results of these tests with moistened sugars inoculated with the bacteria are given and discussed below.

In the descriptions, the growth on sugar agar is given first, as the most characteristic, as well as the most vigorous, growths were obtained on this medium.

BACILLUS A.

This is a very small form, averaging 2 microns in length on the sugar agar, and 1.6 microns on carrot and in glucose bouillon. On agar and in solid media the bacilli are usually single, but they form extensive chains in the liquid cultures. The miscoscopic appearance is shown in Fig. 3, from sugar agar, Fig. 4, from carrot, and Fig. 5 from glucose bouillon. No spores were obtained in any of the media, although the bacillus grew very feebly on some and in others was allowed to dry out for several months.

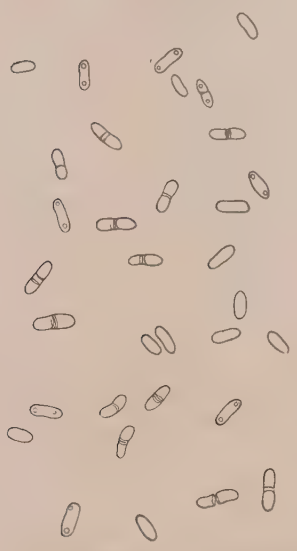

Fig. 3. Bacillus A, from sugar agar. The cells are separate and are in active division. There are no spores. Magnified 2,000 diameters.

SUGAR-PEPTONE 1.5% AGAR.—Two form of colony are found on these agar plates, depending upon whether the colony is submerged or not. The submerged form is approximately circular, while the surface colonies are amoeboid-rhizoid; both forms are shown in Fig. 6 magnified three diameters, a large surface colony rather less than natural size is illustrated in Fig. 7. That the two are merely forms of one and the same growth is proved by the fact that one side of a circular colony occasionally reaches the surface and then it branches out into the characteristic rhizoid growth; this can be seen in Fig. 6; also a double series of plates, one started from a single circular colony, the other from a single amoeboid-rhizoid, gave approximately the same proportion of circular and amoeboid colonies. The amoeboid-rhizoid colony has an umbonate surface elevation, the clear center being raised, while the arms radiating from it are flat. Under the low powers of the microscope the colony appears to have a finely granular

structure; the margin is entire. The texture of the colony is porcelaneous, the center being glistening, while the margin is somewhat dull.

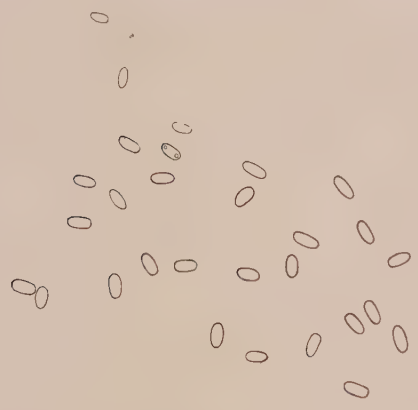

Fig. 4. Bacillus A, from growth on carrot. The cells are isolated and no spores are seen. Magnified 2,000 diameters.

GLUCOSE NUTRIENT 1.5% AGAR.—Very similar colonies to those on the sugar-peptone agar, though not quite so vigorous.

NUTRIENT AGAR.—Very poor growth, small circular plates formed after several days.

An attempt was made to cultivate this form on gelatin media in the ice chest, but growth was almost inhibited at this temperature—about 17° C.

CARROT.—Very quick growth, with a fine enamel-like, smooth, glistening appearance. Edges colorless, remainder of growth cloudy.

TURNIP.—As on carrot.

POTATO.—Very rapid. A rough growth, raised in reticulate manner, of a dirty yellow color. This is shown in Fig. 8, reproduced from a photograph. The recticulate growth later passed over into an enamel, like that on carrot, the difference appearing to be due to the moistness of the part of the substratum upon which the bacillus is immediately growing.

SUGAR-PEPTONE AGAR STAB.—A nodose growth, faintly cloudy. Confined to the agar around the stab, and on the surface. A drawing of this is reproduced in Fig. 9.

SUGAR-PEPTONE SOLUTION.—In two to three days a milky fluid is produced with a slight scum. The analyses of these solutions are given later. No gas evolution is noticeable. The bacilli are in chains (Fig. 5).

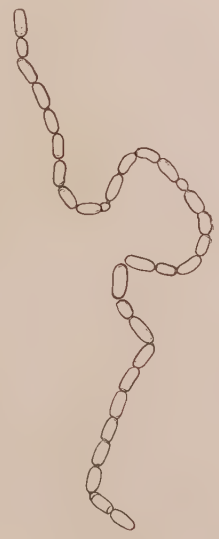

Fig. 5. Bacillus A, from sugar solution. The cells are in chains, as was the case in sugar solution in all the forms examined. Magnified 2,000 diameters.

GLUCOSE NUTRIENT BOUILLON.—A slight cloudiness which does not develop into milkiness.

NUTRIENT BOUILLON.—Very slight cloudiness.

Fig. 6. Enlarged photograph, showing the two forms of colony met with on sugar agar in Bacillus A. One of the circular colonies is seen developing an amoeboid outgrowth on one side. These colonies were three days old. Magnified 3 diameters.

Some interesting tests were made showing the resistance of this bacillus to dessication. A number of plates were allowed to dry gradually in the incubator at a temperature of 33° C. They were then kept at this temperature and tested from time to time. Up to the conclusion of the trials, a period of fifteen months (from January, 1908, to April, 1909), all the plates gave numerous colonies. This high resistance to a very unfavorable method of dessication is of greatest importance in connection with the continued existence of the bacillus in factories. It is clear that even this form, which does not possess spores, is capable of existing

an indefinite period, dry, on the walls and floors and in various containers and be ready to infect and start fermentation in any sugars or sugar-containing liquids it may be introduced into.

DIPLOBACILLUS B.

This is a considerably larger form than the preceding, averaging about 3 microns in length and 1.7 microns in breadth. Large granules are distinctly visible (Fig. 10). The bacilli on the agar

Fig. 7. One of the amoeboid colonies of Bacillus A, from a photograph, rather less than natural size. This colony is six days old.

plates are constantly arranged in pairs; in the liquid cultures there was a tendency to chain formation, but the pairs could still be made out distinctly. The spores are usually terminal, but often central. This bacillus is by far the most easily distinguished of the five investigated, being recognizable among them by its microscopic features alone.

SUGAR-PEPTONE AGAR.—Three forms of colony can be distinguished, depending on their position, as regards depth, in the agar. The colonies on the surface are definite and circular in form, they are enamel-like in appearance and somewhat yellow in color, the surface elevation is pulvinate. With the low power of the microscope the colonies appear homogeneous, with strong

refraction, they are finely granular. The older colonies tend to become somewhat reticulate. The margin is entire. The second type of colony is submerged; it is similar to the preceding, being definite and approximately circular, but it is white and more transparent. Both types of colony can be seen in Fig. 11, from a photograph, slightly less than natural size. Both these forms are decidedly small, seldom exceeding 10 mm. in diameter. At the bottom of the agar, on the glass, irregular, large general, white clouds are formed. In these it is difficult to see any vegetative forms, spore formation being universal.

Fig. 8. Growth of Bacillus A on carrot. On the solid media this bacillus produced either a glistening, enamel-like growth or the slimy corrugated growth shown in the figure, according to the moistness of the substratum. Very similar growths were formed by the bacilli B, C, and D; the bacillus E produced an entirely different growth which is shown in Fig. 21. Magnified 3 diameters.

Fig. 9. A drawing of the nodose growth produced in the agar stab by Bacillus A. Similar growths were produced by all the bacteria described. Natural size.

GLUCOSE NUTRIENT AGAR.—Practically the same as the preceding.

NUTRIENT AGAR.—Colonies very similar, but less homogeneous. Spore formation very abundant.

CARROT, TURNIP, POTATO.—The same enamel-like and rough growths, that were described for A. See Fig. 8.

SUGAR-PEPTONE AGAR STAB.—Nodose growth, very like A. See Fig. 9.

SUGAR-PEPTONE SOLUTION.—In five days a thin cloudy appearance is obtained, with a noticeable coagulum-like precipitate. No gas formation.

NUTRIENT BOUILLON, GLUCOSE NUTRIENT BOUILLON.—A thin cloudiness after several days.

Tests of the vitality of the *Diplobacillus* B were also made in a similar manner to those with the bacillus A; agar plates were dried and kept at incubator temperature and tested from time to time. At the close of the experiments, there was no apparent diminution of vitality. The longest test extended over a period of ten months, from June, 1908, to April, 1909.

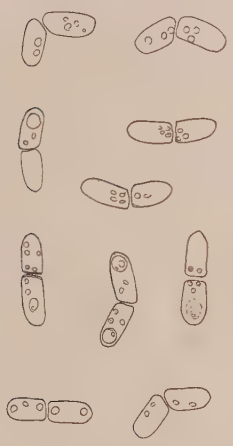

Fig. 10. Diplo-bacillus B, from sugar agar. The cells are large (comparatively) and are always met with in pairs. Granules are distinctly visible. Magnified 2,000 diameters.

BACILLUS C.

Microscopically this is rather a larger bacillus than A, measuring approximately 4 microns in length by 1.2 microns in breadth. The bacilli occur singly on agar, but, as with other forms, are found in chains in the sugar solution. Spores are common in all the media, but less so in sugar solution than on agar; they are usually central, but are occasionally terminal. Fig. 12 from sugar agar shows the general amount of variation met with in the size and shape of bacilli and spores.

SUGAR-PEPTONE AGAR.—Colonies very variable in form; they may be circular and very definite, a general cloud effect without any definite shape, or distinctly amoeboid. The photographs reproduced in Figs. 13 and 14, give some idea of the variation that is met with. The three general forms mentioned are all produced on the surface of the agar. Sometimes the central part of a cloudy colony will later give rise to an amoeboid colony within the boundaries of the older one. The amoeboid and cloud colonies are indefinitely large. All are slightly umbonate in surface elevation. The refraction of all is weak; the colonies are homogeneous and homochromous; somewhat translucent. Under the low power of the microscope the structure appears granular; the margin is entire.

GLUCOSE NUTRIENT AGAR AND NUTRIENT AGAR.—The colonies are much smaller than on sugar-peptone agar, not more than 15 mm. in diameter. There are no cloud-like colonies, but they are circular or somewhat frondose. The colonies are convex in surface elevation, in appearance opaque and glistening. Under the low power they appear finely granular; the margin is entire or with small, widely spaced projections.

SUGAR-PEPTONE SOLUTION.—Decidedly milky growth in two days. The gum formation is distinctly high; in one case 1.28

grams from 11 grams sucrose in seven days. A neutral litmus sugar solution was distinctly reddened in twenty-four hours, by the next day the litmus had been carried down by the gum.

Fig. 11. Photograph, rather less than natural size, of the colonies formed by Bacillus B on sugar agar. These are distinctly small, and circular. The colonies are six days old.

GLUCOSE NUTRIENT BOUILLON AND NUTRIENT BOUILLON.—A slight, dirty scum on the surface after several days.

SUGAR-PEPTONE AGAR STAB.—Nodose, very like Bacillus A. (Fig. 9).

POTATO, CARROT, TURNIP.—Enamel, passing to corrugated growth, very like Bacillus A (Fig. 8).

BACILLUS D.

Microscopically this form is very similar to Bacillus C, the average dimensions of bacilli taken from sugar agar being approximately 3.7 microns in length by 1.2 microns in breadth. The spores are usually central, as shown in Fig. 15, they are not commonly seen in the sugar solution cultures, but are abundant in all the other media. Long chains are met with in the sugar solution, these are shown in Fig. 16; on the other media the bacilli are found singly. A curious appearance is presented by preparations stained with Ziehl-Nielson carbolfuchsin, which is shown in Fig. 17. The bacilli are gathered together in masses and appear to consist of a faintly stained central part, with a very brightly stained circular body at each end; the appearance resembles very strongly the 'bi-polar' staining described for certain pathogenic bacteria, notably *Bacillus pestis* (plague bacillus),* though in the present case double staining was not used. A comparison of Fig. 17 with Figs. 15 and 16, drawn to the same magnification from fresh preparations, would seem to show that the bi-polar structure does not represent the whole bacillus cell; and occasionally a faint trace of a swollen outer wall can be made out, or rather the outline of a general mass in which the groups of bacilli are lying. It seems possible that the central body represents the spore, the two brightly staining bodies the degenerated protoplasm of the remainder of the cell, while the walls of the cells have swollen up and become confluent with those of other cells lying close at hand. This peculiar effect was not met with in the other bacteria stained in the same way, and was always

Fig. 12 Bacillus C, showing range of bacterial cells and spores met with on sugar agar. Spores are of frequent occurrence, and are usually central. This is a larger form than Bacillus A. Magnified 2,000 diameters.

* Jordan: General Bacteriology, 1908, p. 290.

obtained with D, so that it would appear to have some diagnostic value.

SUGAR-PEPTONE AGAR.—Two forms are usually met with, de-

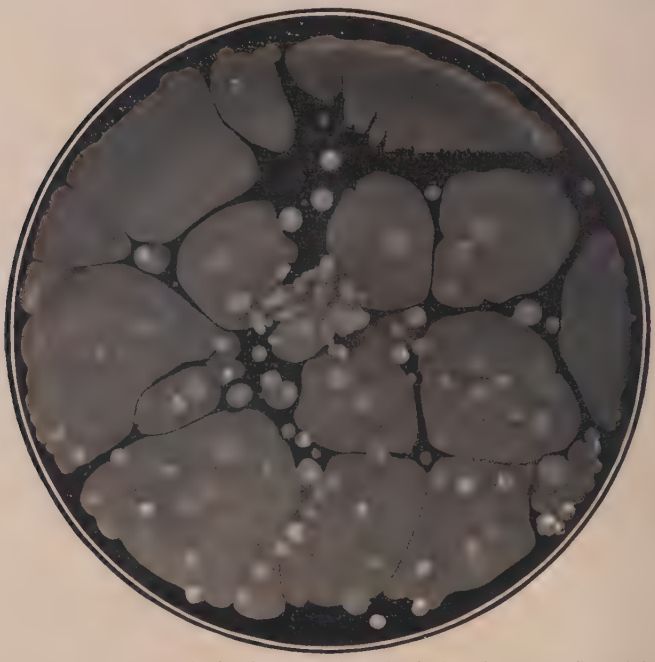

Fig. 13. Photograph showing a number of colonies of Bacillus C, slightly less than natural size. Some of the smaller circular colonies are seen, as also the indefinite, cloud-like colonies referred to in the text. Compare with Fig. 14.

pending on whether the colony is submerged or on the surface of the agar. The submerged colonies are approximately circular; those on the suface are intermediate between rhizoid and amoeboid. The photograph, slightly less than natural size, reproduced in Fig. 18, shows the two forms of colony on the same plate and also shows a circular colony developing at one side a rhizoid-like outgrowth. This form differs therefore from C in not possessing the same variety in its surface colonies. Both forms of colony attain a large size. The rhizoid colonies are umbonate in surface elevation; in both forms the refraction is strong and they appear coarsely granular; the edges are very slightly erose. In appearance the colonies are opalescent.

NUTRIENT AGAR AND GLUCOSE NUTRIENT AGAR.—The colonies are much smaller than on the sugar-peptone agar, but the same amoeboid-rhizoid and circular forms occur; here, however, some

of the circular colonies are on the surface. They are considerably more opaque than on sugar agar and are glistening. Under the low power of the microscope the colonies appear more finely granular; they are also more highly refractive.

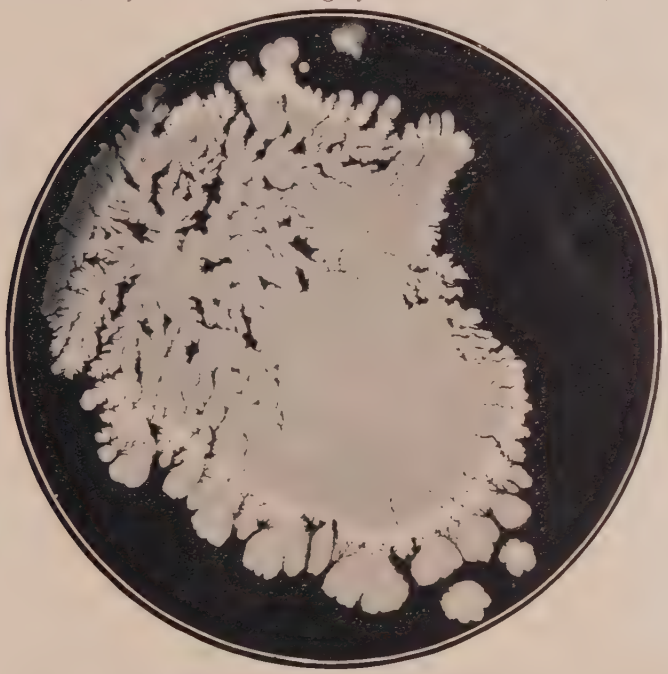

Fig. 14. Photograph showing a large colony of Bacillus C on sugar agar, growing under the same conditions as those shown in Fig. 13. The colony is six days old.

Fig. 15. Bacillus D from a plate colony on sugar agar. This shows about the range of the bacterial cells and spores usually met with. Magnified 2,000 diameters.

SUGAR-PEPTONE SOLUTION.—Rapid growth takes place, without the evolution of gas. The solution is decidedly milky in appearance by the second day after inoculation. The formation of gums is high, one result giving 1.11 grams from 10 grams sugar in seven days; D is thus of the same order, as regards gum formation, as the forms A and C. A neutral litmus sugar solution was reddened within twenty-four hours; later, as with Bacillus C, the litmus is precipitated.

NUTRIENT BOUILLON AND GLUCOSE NUTRIENT BOUILLON.—A faint cloudiness

Fig. 16. Bacillus D from sugar solution. The figure shows the long chains, met with in this medium, in which it is difficult to make out the individual cells. Spores are less abundant here than on the agar cultures.

is noticeable in two days and at the same time a slight scum is formed. This later sinks, giving a small precipitate.

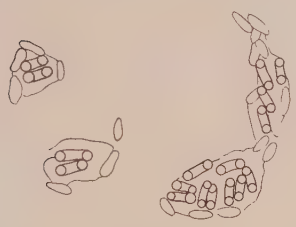

Fig. 17 Bacillus D, showing the curious bi-polar effect produced in this form by carbol-fuchsin staining. This effect is discussed in the text. Magnified 2,000 diameters.

SUGAR-PEPTONE AGAR STAB. — Very similar to the other forms described (Fig. 9).

POTATO, CARROT, TURNIP.—Enamel or corrugated growth as in the three preceding forms (Fig. 8).

BACILLUS E.

This form is very similar microscopically to C and D, although it frequently appears slightly curved. As with the other forms,

Fig. 18. Photograph of colonies of Bacillus D as they appear on sugar agar. This shows the different forms of colony, even on the same plate. The colonies are six days old. Rather less than natural size.

long chains are found in the sugar-peptone solution. The cells average approximately 3.5 microns in length, by 1 micron in

breadth; it is thus rather longer in proportion to its breadth, than the other forms mentioned. Spores are produced in all media, but, as usual, less abundantly in the sugar peptone solution.

SUGAR-PEPTONE AGAR.—Colonies smaller than those of the forms A, C, and D, seldom reaching 10 mm. in diameter. Both surface and submerged colonies are approximately circular (Fig. 20). The surface colonies are capitate in surface elevation. They are clear in the center with a white, faintly translucent border. Occasionally the border spreads out irregularly for a considerable distance from the original circular growth. The refraction is strong. Under the low power of the microscope the colonies appear distinctly finely granular, the edges are entire.

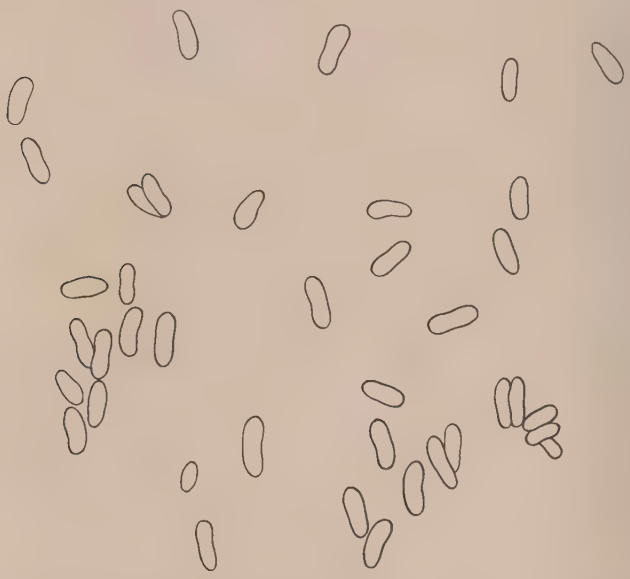

Fig. 19. Bacillus E, showing the bacterial cells as they occur in the sugar agar colonies. Magnified 2,000 diameters.

GLUCOSE NUTRIENT AGAR.—The colonies are for the most part circular, some are irregular; they are quite small, the circles being only 1 to 2 mm. in diameter. They are flat in surface elevation. Refraction is strong. Under the low power they are distinctly finely granular; the edges are irregular. The colonies are yellowish, opaque and glistening.

NUTRIENT AGAR.—In appearance similar to those on glucose nutrient agar, but even smaller. They are white, not yellowish. Under the low power the colonies are distinctly marmorate, the

segments being very regular, somewhat square and well separated; the edges are erose.

Fig. 20. Photograph showing the colonies of Bacillus E on sugar agar plates. The colonies are small and generally circular, they have marked, raised, clear centers. These colonies were thirteen days old. Rather less than natural size.

SUGAR-PEPTONE SOLUTION.—The solution becomes cloudy in twenty-four hours, but there is no further change to milkiness. A coagulum is formed later. Gum formation is vey slight, amounting, in ten days, only to 0.17 grams from 10 grams sucrose. The production of gum, therefore, sharply distinguishes between the forms A, C, and D, where it is abundant, and B and E, where it is so slight as to be almost negligible. Litmus sugar solution is reddened.

GLUCOSE NUTRIENT BOUILLON.—A marked, somewhat stringy, scum is formed in twenty-four hours.

NUTRIENT BOUILLON.—Only a slight cloudiness is produced even after several days.

SUGAR-PEPTONE AGAR STAB.—Very like the other forms examined (Fig. 9).

POTATO, CARROT, TURNIP.—Rapid growth of a whitish, dry in-

crustation, with irregular eruptions. This is illustrated in Fig. 21, from a photograph at a magnification of three diameters, and is quite distinct from the moister, somewhat slimy, growth met with in other forms. The difference does not appear to be due to any variation in the moisture of the substratum.

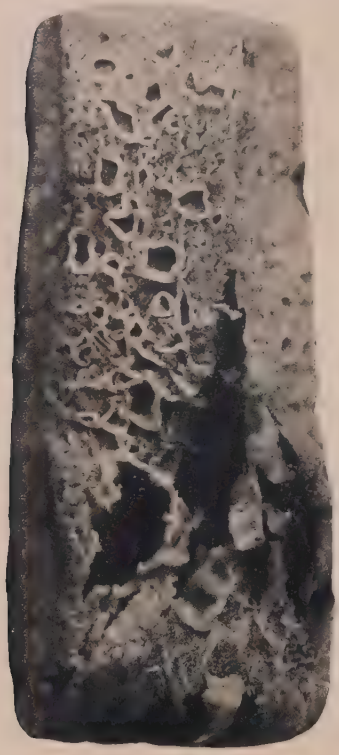

Fig. 21. Photograph of the growth of Bacillus E on carrot. This growth is dry and eruptive, quite distinct from that produced by the other forms described. Compare Fig. 8.

GENERAL.

As flagella have not been demonstrated in any of the forms described, they would be placed in Migula's genus *Bacterium* rather than *Bacillus*. It is noticeable that all five forms give the same growth in the agar stabs, while the first four also give indistinguishable growths on potato, carrot and 'turnip.' B and E are readily distinguishable by the small gum production in sugar solution, and by their limited, circular colonies, while E is sharply marked from the others by its marked capitate colonies on sugar-peptone agar with their large clear centers. A is readily separated from C and D by its microscopic characteristics, including the absence of spores, by its extremely weak growth on nutrient agar, and by its two definite forms of colony on sugar-peptone agar. C and D are much alike in their cultural and microscopic characteristics, C is, however, much more polymorphic in its colonies than D, although both are variable; the clearest distinguishing feature is the absence of the shapeless, cloud-like colonies and of circular surface colonies from D; possibly also the curious bipolar staining with carbol-fuchsin is characteristic of D; the two forms are very different in their action on moist sugars, as is shown by the tables below; in solutions their action is practically identical.

Action in Liquid Medium.

BACILLUS A.

	Days.	Sucrose, Grams per 100 cc.	Invert Sugar, Grams per 100 cc.
Control	5	10.00	....
Inoculated	5	1.06	7.08

There was also formed 0.522 gram gum, i. e., ash free alcoholic precipitate.

This bacillus is similar in its action to Bacillus D, described below, in that the sum total of the cane sugar and invert sugar formed does not represent approximately the amount of cane sugar initially present; evidently products other than invert sugar and gums are formed by its action.

BACILLUS B.

	Days.	Sucrose, Grams per 100 cc.	Invert Sugar, Grams per 100 cc.
Control	5	10.19	Trace
Inoculated	5	6.84	3.16

A trace only of gums was formed.

The inversion due to this organism in liquid culture is small compared with that of the bacteria A, C and D, but resembles that of Bacillus E. This is correlated with its action on commercial sugars, where also the destruction of sucrose was comparatively small in comparison with the action of the bacteria A and C. Nearly all the sucrose originally present is recoverable either as sucrose or as invert sugar.

BACILLUS C.

	Days.	Sucrose, Grams per 100 cc.	Invert Sugar, Grams per 100 cc.
Control	2	10.36	....
Inoculated	2	4.69	4.79
Control	5	10.36	....
Inoculated	5	0.00	8.48
Control	5	11.00	0.00
Inoculated	5	0.18	10.85
Control	10	11.00	0.00
Inoculated	10	0.09	10.51

In two days there were formed 1.02 grams of ash free gums and in five days 1.58 grams. In another experiment 1.28 grams gums were produced in seven days, so that the amount of gums formed appears distinctly variable.

It would also appear that the action of the bacillus is completed by the fifth day, although there are large amounts of sugars

available for growth. This point is again referred to under Bacillus D, where the same inhibition of bacterial activity is noted.

The action of this organism seems to differ from that of the other gum forming types A and D in that the total of reducing sugars and gums formed is equal approximately to the original amount of cane sugar present; with the forms A and D, there is evidently a production of other compounds besides invert sugar and gums.

BACILLUS D.

	Days.	Sucrose, Grams per 100 cc.	Invert Sugar, Grams per 100 cc.
Control	5	10.01	....
Control	10	10.01	....
Inoculated	5	0.38	7.62
Inoculated	10	0.37	7.57

In seven days 1.108 grams gums were formed from 100 cc.

The action of this organism is comparable with that of the form A, as not only is the sucrose nearly completely inverted, but a considerable portion of the sucrose has entirely disappeared as sugar.

It was noticeable with this Bacillus as with C, that, although the whole of the cane sugar is rapidly inverted, the destruction of the glucose formed does not progress beyond a certain stage; thus in the table above, although a considerable quantity of glucose has disappeared by the fifth day, there is practically no further loss by the tenth day. It seemed probable that this inhibition was due to some of the products of bacterial activity. The production of acids had been proved in the solutions and an experiment was designed to test whether these were the products that prevented the further destruction of the glucose. If so, the destruction should go on much further, in fourteen days, in flasks containing calcium carbonate, where acids would be neutralized as formed, than in the flask where the acids were allowed to accumulate. Accordingly, to 100 cc. of a 10 per cent. solution of sugar with nutrient salts 0.1 gram calcium carbonate and 0.2 gram calcium carbonate were added; these, together with a check, were inoculated as in the other experiments.

The experiment might also throw some light on the question as to whether the reaction of the medium has any effect on the growth of the bacterium, as it has not infrequently been stated that alkaline sugars keep better than those of neutral or acid reaction. The results obtained were:

	Days.	Sucrose, Grams per 100 cc.	Invert Sugar Grams per 100 cc.	Gums, Grams per 100 cc.
Check	14	0.30	8.16	0.813
0.1% Cal. Car...	14	0.29	8.24	1.010
0.2% Cal. Car...	14	0.24	8.37	0.748

showing that the alkaline reaction of the medium has no effect on the growth of the bacillus.

The results are very close indeed and also very similar to those of the previous experiment given above. It is thus certain that it is not the acids produced that inhibit the activity of the bacteria, but probably some of the other by-products. Further investigation of these products should prove an interesting piece of research, which we were compelled to abandon.

It is noticeable that the amount of gum produced is distinctly variable, even in the same solutions kept under identical conditions and where the figures for the sugars are practically identical. The same variability was pointed out with the Bacillus C.

BACILLUS E.

	Days.	Sucrose, Grams per 100 cc.	Invert Sugar, Grams per 100 cc.
Control	5	10.36	....
Inoculated	5	9.40	0.81
Control	10	10.36	....
Inoculated	10	5.20	4.80

In ten days .171 gram of gums was formed per 100 cc.

The invertive action of this organism is small compared with that due to the forms A, C, and D, and it is comparable with that noted with the form B.

Summarizing these results, it appears that a faculty of rapidly inverting sugar is associated in liquid cultures with the formation of gums. With forms A and D is also associated a loss of material as sugars, which does not occur with the form C.

NATURE OF THE GUM FORMED.

The gum produced by the forms A and C was examined. On heating with hydrochloric acid it was hydrolized to levulose; with nitric acid it was oxidized to oxalic acid; basic lead acetate and lime water gave no precipitation; a precipitate was formed with ammoniacal lead acetate and with baryta water; the gum had very little action on Fehling's solution; it was found impossible to determine the opticity of the gum in aqueous solution, owing to the turbidity of the solution. These properties are identical with those found by Greig Smith to be possessed by levulan, the name he gave to the gum formed by *B. levaniformans*, and this gum is probably identical therewith, so that the production of a gum with the properties of levulan cannot be held as characteristic of one particular bacterium.

Action on Commercial Sugars.

The methods of sterilization and inoculation are described on p. 8. See also Fig. 1, frontispiece.

BACILLUS A.

	Days.	Water %	Sugar % on dry Weight.	Invert Sugar % on dry Weight.
Control	7	2.75	97.2	....
Inoculated	7	2.61	95.9	....
Control	14	2.76	97.6	0.70
Inoculated	14	2.26	95.4	1.92
Control	21	2.82	97.9	1.04
Inoculated	21	2.34	94.3	3.55

There has been a steady drop in the polarization of the sugar accompanied by a corresponding, but smaller, increase in the amount of invert sugar; the sucrose destroyed during the first week amounted to 1.3 per cent., after two weeks to 2.2 per cent., and in three weeks to 3.6 per cent.; after two weeks the invert sugar had increased by 1.22 per cent., and in three weeks by 2.51 per cent.; this organism which had the most pronounced effect on sugar in liquid medium is also the one that (under like conditions) causes great deterioration in commercial sugars, being equalled only by the form C.

In the first experiment with this bacillus, a smaller amount of moisture was obtained in the sample than was intended, and, owing to the flasks not being covered with rubber tissue, some of the water present evaporated. The results obtained in this experiment were:

	Days.	Water %	Sugar % on dry Weight.	Invert Sugar % on dry Weight.
Control	7	0.99	96.2	0.85
Inoculated	7	0.99	95.8	1.10
Control	14	0.68	97.3	....
Inoculated	14	0.58	96.8	....

The third samples were then placed in a moist chamber at air temperature, and analyzed at the end of thirty days with the following results:

	Water %	Sugar % on dry Weight.	Invert Sugar % on dry Weight.
Control	3.43	97.5	0.81
Inoculated	4.31	95.3	1.32

These experiments again demonstrate the dependence of bacterial change on a sufficient quantity of water; although many

times more bacteria would be present than should ever be found in practice, the fall in polarization with the comparatively dry sugars in the first seven days was small. When the water present fell below 1 per cent., as it had by the end of the first seven days, there was no further deterioration.

BACILLUS B.

	Days.	Water %	Sugar % on dry Weight.	Invert Sugar % on dry Weight.
Control	7	3.80	96.9	2.12
Inoculated	7	4.04	96.2	2.37
Control	14	3.82	96.8	2.27
Inoculated	14	4.02	95.6	3.03
Control	21	4.02	96.7	2.34
Inoculated	21	3.98	95.4	3.09

Although a larger percentage of water was present than in the experiment with Bacillus A, the destruction of sugar due to this form was small. It will be seen that at the end of one week the loss of sugar equalled 0.7 per cent., with an increase in invert sugar of 0.25 per cent.; at the end of two weeks the figures show a loss of sugar of 1.2 per cent., and a gain of invert sugar of 0.76 per cent.; and in three weeks loss of sugar amounted to 1.3 per cent., and increase of invert sugar to 0.75 per cent., practically no change from the second week. This weak deteriorating action on raw sugars can be correlated with the slight inversion brought about in the solutions of sugar (p. 25).

BACILLUS C.

	Days.	Water %	Sugar % on dry Weight.	Invert Sugar % on dry Weight.
Control	7	3.31	98.7	0.59
Inoculated	7	3.05	96.0	1.83
Control	14	3.54	98.8	0.56
Inoculated	14	3.66	95.1	2.55
Control	21	3.13	98.6	0.56
Inoculated	21	3.29	94.1	2.92

The deterioration due to this form is large and similar to that shown by the form A. The loss of sucrose in one week amounts to 2.7 per cent., compared with the control, in two weeks to 3.7 per cent., and in three weeks to 4.5 per cent.; for the same periods the gains in invert sugar were 1.24 per cent.. 1.99 per cent., and 2.36 per cent. This is the largest deterioration recorded in the experiments, but as there was rather more water present here than in the experiment with Bacillus A, it would be better to regard these forms as approximately equal in deteriorating capacity. The action of C on sugar corresponds with its invert-

ing action on sugar in liquid medium, and is correlated with its gum forming power.

BACILLUS D.

	Days.	Water %	Sugar % on dry Weight.	Invert Sugar % on dry Weight.
Control	7	2.57	98.0	1.32
Inoculated	7	2.38	96.8	1.87
Control	14	2.20	97.9	1.29
Inoculated	14	1.68	96.2	2.05
Control	21	2.20	97.9	1.26
Inoculated	21	2.09	96.1	2.36

The deterioration in this instance is not so pronounced as with the other gum forming types. In one week the sugar destroyed amounted to 1.2 per cent. as compared with the control, in two weeks to 1.7 per cent., and in three to 1.8 per cent.; for the same periods the increases in invert sugar were 0.55 per cent., 0.76 per cent. and 1.10 per cent. It would seem that the deterioration has stopped apparently at the end of fourteen days. This may be due to the smaller amount of water present preventing further deterioration after a certain point has been reached. However, a further experiment with this form, under varying conditions as regards moisture, also showed that it has not an equally destructive action on sugars to the forms A and C. This experiment is described on p. 32.

BACILLUS E.

	Days.	Water %	Sugar % on dry Weight.	Invert Sugar % on dry Weight.
Control	7	2.70	97.8	0.62
Inoculated	7	2.64	97.6	0.61
Control	14	2.58	97.7	0.68
Inoculated	14	2.66	97.0	1.18
Control	21	2.91	97.7	0.72
Inoculated	21	2.87	96.5	1.58

With this form, as with the other non-gum forming type, the deterioration is comparatively small; the sucrose destroyed in three weeks amounts only to 1.2 per cent., with an increase of 0.86 per cent. in the invert sugar; these are very similar results to those that were obtained with the bacterium B.

Influence of External Conditions.

The two most important conditions affecting the growth and activity of the bacteria are the temperature and the amount of

moisture. As this work was planned primarily with the idea of comparing the different bacteria found in Hawaiian sugars, no very extensive tests were made to work out the exact influence of external conditions on any one form, the plan being rather to test each bacterium under approximately the same conditions as all the others.

TEMPERATURE.

Growth on all media was found to be many times more rapid at incubator temperature (33° C.) than at room temperature (25° C.); thus colonies that would be 5 to 10 mm. in diameter in two days when kept in the incubator, would hardly be visible in less than four days in the room.

An experiment, already mentioned on p 28, with Bacillus A will serve to illustrate the influence of temperature on the rate of deterioration. In this case it was found that by the end of the second week after inoculation, the sugars had become dried out to such an extent that no further deterioration had taken place. The two remaining sugars (one control and one inoculated) were, therefore, taken from the incubator and kept at room temperature in a saturated atmosphere for a further period of thirty days. In this time the quantity of water in the inoculated sugar increased to over 4 per cent. The total deterioration in this sugar then amounted to 2.2 per cent., with an increase of 0.51 per cent. in the invert sugar. If we deduct from this the deterioration that took place during the first week in the incubator, we find the deterioration, that took place during the thirty days at room temperature, amounts to 1.8 per cent., and the increase of invert sugar to 0.26 per cent. Comparing this with the second experiment carried on at incubator temperature, given on p. 28, we see that with the latter the deterioration amounted to 3.6 per cent., with an increase in invert sugar of 2.51 per cent. in three weeks, although the percentage of water was only 2.34. Thus an increase in temperature of 8° C. has more than doubled the rate of deterioration, even leaving out the difference in water content, which is difficult to estimate accurately.

Cultures of Bacillus A at 17° C. grew so slowly that the colonies were hardly visible after several days. It seems probable that deterioration also would be very slow at this temperature.

MOISTURE.

The experiment just quoted with Bacillus A also throws light on the dependence of the activity of the bacteria on the moisture present in the sugars. At the end of the first week the water content of the sugars was reduced to about 1 per cent., and a

slight deterioration had taken place. After this there was no further loss of sugar. The inference is fairly certain that the activity of the bacillus is entirely inhibited when the moisture content of the sugar sinks to an amount which is somewhere in the neighborhood of 1 per cent.

To test this and also to test whether the rate of deterioration would continue to increase with increasing moisture content, a more definite experiment was carried out with Bacillus D. Six flasks, each containing 100 grams of sugar, were all sterilized under precisely the same conditions. Five of them, numbered 2, 3, 4, 5 and 6, were then sprayed with sterile water for 10, 20, 30, 40, and 30 seconds respectively, it having been calculated that the atomizer would deliver about 10 cc. of water in 10 seconds. Numbers 1, 2, 3, 4, and 5 were then sprayed with an active liquid culture of the bacillus each for 10 seconds. Each of the flasks, except No. 6, which served as a control, thus received an equal inoculation, while they should show a moisture content varying from 1 to 5 per cent.; the control would have 3 per cent. water.

The analyses were made after fourteen days with the following results:

No.	Water %.	Sugar % dry Weight.	Invert Sugar % dry Weight.
1	1.35	97.5	1.54
2	1.84	97.5	1.51
3	3.21	97.0	1.60
4	4.06	96.5	1.63
5	4.70	96.0	1.70
6	3.18	97.7	1.32

The results show very clearly that up to 5 per cent. of water, the rate of deterioration shows a steady increase with the increase of moisture content; with the lowest percentages of water, the deterioration is very small, though the coincident increase of invert sugar would show that it is real. With the increasing percentages to approximately 3, 4, and 5, the increasing deterioration is marked and very regular.

Another interesting feature brought out by these results is the increasing destruction of total sugars calculated on dry weight with the increase of moisture content. With numbers 1 and 2 there is apparently no loss of total sugar as compared with those of the control; with number 3 there is a distinct, though small, loss, which increases to nearly 1 per cent. with 4, and to over 1 per cent. with 5. It will be noticed that in no case, in the series of tables given above, does the increase in invert sugar account for the whole of the sucrose lost, the difference of course being accounted for by other products of bacterial activity.

Another point of interest that requires further investigation is

whether there is a definite maximum of deterioration for each bacillus, for each percentage of water, or whether the deterioration will go on indefinitely, merely varying in rapidity according to temperature and moisture conditions. The probability is that it will go on indefinitely, but there is also the possibility that an accumulation of by-products might inhibit further activity when a certain point has been reached. It is certainly curious that bacteria B and D both showed practically the same deterioration at the end of the third week's incubation, as at the end of the second, though this might have been due to errors of experiment.

GENERAL CONSIDERATIONS.

The main point brought out in this investigation, is the dependence of deterioration on the influence of bacteria acting in the presence of a sufficiency of water and at a temperature favorable for their development. Consequently, if sterile sugars could be made, the chief source of harm would be eliminated. Those acquainted with the details of manufacture of cane sugar, will at once see how difficult it would be to work under aseptic conditions and with the complete absence of bacteria. On the other hand, any one conversant with different factories and different methods of manufacture would be able to select certain factories where a contaminated sugar or one approximately sterile would be expected; for example, a sugar resulting from a "first-sugar and molasses" process, where the intermediate products are mostly in closed containers and which lasts in all not more than six or seven days, would be expected to be less infected than one obtained by a process of repeated boiling in which the intermediate products are exposed in open containers for months. It is intended, as soon as possible, to make a bacteriological survey of a number of factories in these islands, and already, where occasion served, a little preliminary work has been carried out in a factory representing the older type of sugar house design. It was found that the raw juice contained a very large number of bacteria of which a small number survived the passage of the juice through the heaters. Those which survived this treatment were unaffected by the passage through the evaporators and pans, and appeared in the syrups and massecuites. At the centrifugals, at this factory, a very little water was used, mainly to wash down the spouts of the pug mills and also the centrifugal spindles. This water was found to contain, even in one cubic centimeter, an uncountable number of organisms, and the resulting sugar was so infected that it contained upwards of 3,000 bacteria per gram. Infection at this point might partly be eliminated by taking steps to work here under more aseptic conditions, as by not allowing

water to accumulate in the containers, by the use of none but condensed water, and by not letting water remain in the small containers used to store it at the centrifugals. It was also observed that the surface layers of all massecuite and molasses tanks contained very large numbers of bacteria, so that the re-introduction of low sugars must also be considered as a cause of re-infection.

The moisture and temperature conditions are also to a certain limit under control. In some factories in these islands, it is the custom to bag the sugar directly as it is discharged from the centrifugals. The bagged sugar then contains a maximum of moisture and is at high temperature, which in the interior of the bag will be maintained for some time. In the beet sugar factories on the continent of Europe, great attention is paid to a proper cooling of the sugar before placing it in the containers, and packing the sugar while hot and before it has had opportunity to part with its surplus moisture is held to be a frequent cause of subsequent deterioration.

At the last two meetings of the Planters' Association, storage and transportation have been very completely discussed; the dependence of sweating* on badly constructed warehouses has been clearly brought out; and the data brought forward here, showing the dependence of bacterial action on a sufficiency of moisture, should be a strong argument in favor of constructing the warehouses tight, especially in those districts where the climatic conditions are such that a damp atmosphere results.

In the discussions that have recently taken place, diametrically opposite views have been expressed regarding the advisability of opening the hatches or of keeping them closed during the voyage round the Horn. Cases have been quoted where sugars shipped with the hatches kept closed arrived in good condition, and again the reverse has happened. We think that the changes in temperature during the voyage have a certain bearing on this question. The amount of moisture held in the atmosphere increases as the temperature rises. On leaving these islands and going south, the air in the hold reaches a high temperature. On leaving the tropics, the temperature would fall and moisture will be deposited on the sugar which will, by reason of its hygroscopic nature, take up the water and will "sweat"; hence opening the hatches when traveling with a fall of temperature would probably allow moisture to deposit on the sugar. On the other hand,

* By sweating sugar we understand a sugar which has, owing to improper condition or storage, absorbed so much water as to stain its containers. This condition is, we believe, largely determined by the nature of the adhering film of molasses, and a sterile sugar would be equally likely to sweat with one infected with bacteria. An actual deterioration, as judged by the fall in polarization, would not occur in a sterile sugar, in the light of our experiments.

when traveling northwards, after rounding the Horn, opening the hatches would admit warm air into the hold which would then take up and carry away moisture. If the hatches should then be closed down before a fall of temperature happens, it does not seem likely that damage would ensue.

At the last meeting of the Planters' Association opinions were strongly expressed in favor of the Keen ventilator. This process, we understand, maintains a current of air round the hold, but not in it. In so far as this scheme will tend to keep the hold cool and prevent the temperature rising to that degree where bacterial activity is most pronounced, we think that this scheme is likely to prove of great service. It would also be possible, we think, to extend it to the warehouses used for storage of sugars, building these with double walls, so as to allow for the circulation of a current of air between the walls and at the same time preventing the ingress of moist air during the presence of unfavorable climatic conditions.

These considerations on transport and storage can at best be only palliative, and we are strongly of opinion that the best safeguard against deterioration is the maintenance of the factory itself in as aseptic condition as is possible, the avoidance of the introduction of bacteria, as for instance in the use of unclean water at or about the centrifugals, and the disinfection of the factory during the off season. This last method was highly spoken of by Mr. John Watt of Olaa at the Planters' Meeting of 1907 as effective in removing the causes of deterioration from a badly infected factory.

APPENDIX.

By R. S. Norris.

In the Bulletin, No. 24, of the Division of Agriculture and Chemistry on "The Deterioration of Sugars on Storage," referred to on page 5, an experiment was described which was undertaken "to determine the percentage of water which it is safe to leave in sugars." Separate portions of a sample of moist sugar containing fermenting organisms, were dried in a vacuum so as to contain decreasing amounts of moisture from 1.86 to .29%.

The samples were polarized and put into tightly stoppered bottles. At the end of one and two months they were polarized again, and it was found that in those samples containing more than 1% of moisture, the polarizations were lower than originally.

These samples have been recently polarized again after standing twelve months, and the results further confirm the original conclusion that raw sugars containing 1% or more of moisture are liable to deteriorate on storage. The original table with the further polarizations added is given below:

Per cent. Moisture	POLARIZATIONS			
	Initial.	After 1 month	After 2 months	After. 12 months
.29	96.8	96.7	96.7	96.5
.40	96.6	96.6	96.6	96.5
.47	96.8	96.6	96.6	96.2
.59	96.8	96.6	96.7	96.4
.65	96.4	96.4	96.6	96.2
.74	96.4	96.4	96.5	96.2
.96	96.1	96.0	96.0	95.7
1.04	96.0	95.9	95.7	95.1
1.18	96.0	95.2	95.2	94.6
1.28	95.8	95.0	95.0	94.2
1.36	95.8	95.0	94.7	94.4
1.51	95.5	94.7	94.5	93.8
1.67	95.6	94.2	94.1	93.4
1.80	95.3	93.8	94.0	92.7
1.86	95.15	94.4	94.0	93.1

Notwithstanding that the bottles were closed with rubber stoppers, the sugar was so hygroscopic that the samples had nearly all increased slightly in moisture during the year, which accounts for the lower polarizations of those containing less than 1% of moisture.